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GENETIC POLYMORPHISM AND SERUM LEVEL OF VASCULAR ENDOTHELIAL GROWTH FACTOR ASSOCIATED WITH FIRST TRIMESTER PREGNANCY OUTCOME IN ICSI PROGRAM

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ABSTRACT

VEGF plays an important role in normal physiological processes that require increased vascularization to bring oxygen and nutrients to tissues, including embryonic development. This study determine the relationship between changes in serum level of vascular endothelial growth factor (VEGF) protein and pregnancy outcome in women undergoing ICSI and also the relationship between (VEGFA) single nucleotide polymorphism (rs 3025039) 936 C/T and it's incidence on pregnancy outcome after ICSI. The study was done on 51 infertile women and 34 fertile women with at least 2 live births. Patients categorized as pregnant, non-pregnant and miscarriage. The levels of vascular endothelial growth factor protein were measured by ELISA, the results showed that the levels of vascular endothelial growth factor in control, ICSI patients, pregnant, non-pregnant and miscarriage were (1.99±0.54mg/ml, 2.46±0.64mg/ml, 2.80±0.61mg/ml, 2.21±0.60mg/ml, 2.56±0.30 mg/ml respectively). The significant increase shown in pregnant when compared with control, pregnant p= 0.0004, ($p<0.05$) and also there is significant increase in pregnant when compared with non-pregnant, p=0.0081, ($p<0.05$). The genetic polymorphism VEGFA/rs 3025039 was detected by specific primers. Results of amplification showed that a DNA fragment 198 bp was obtained after electrophoresis on 2% agarose gel. The nucleotide sequence of each amplified fragment was determined. Results of sequencing showed that the genotype frequency in control were CC (85.29%), CT (11.76%), TT (2.94%), in ICSI patients were CC (72.54%), CT (23.52%), TT (3.92%), in pregnant were CC (62.5%), CT (16.66%), and TT (0%), in non-pregnant were CC (70%), CT (25.92%),TT (3.7%) and in In miscarriage

were CC (60%), CT (20%),TT(20%). VEGFA/rs 3025039 polymorphism has a non-significant association with pregnancy outcome in ICSI program. The results of this study shows that increasing level of vascular endothelial growth factor associated with pregnancy also there is no association between 936 C/T polymorphism and pregnancy, implantation failure and miscarriage.

KEYWORDS: Vascular Endothelial Growth Factor (VEGF), rs3025039, ICSI.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a key regulatory molecule that promotes growth of new blood vessels.[1] The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGF (placental growth factor) which is exist in mammalian genomes.[2] VEGF-A gene is located on chromosome 6p21.3 and is encoded by 8 exons separated by 7 introns.[3] A central role of VEGF in fetal and placental angiogenesis was suggested from murine gene knockout studies,[4] where it was shown that mice lacking VEGF expression died in utero due to inadequate vascularization.[5,6]

In human early pregnancy, VEGF is essential for optimal trophoblast proliferation, and the establishment of adequate maternal and fetal circulation[7]. Recurrent implantation failure and recurrent miscarriage are the main clinical phenotypes associated with VEGF deregulation. There are different genetic variants of VEGF, mainly single nucleotide polymorphisms (SNPs), which affects the gene expression or protein structure and has negative impact on human reproduction.[8]

One of these polymorphisms has been reported in the VEGFA gene, which include 936 C/T in 3 ' untranslated region Which is associated with altered VEGF secretion. Several reports investigated the association of VEGFA gene variants with altered VEGF secretion and recurrent spontaneous miscarriage risk.[9,10]

MATERIAL AND METHOD

Subjects

The study was done on both 51 infertile women undergoing ICSI from high institute of infertility diagnosis and assisted reproductive technologies, Al-Nahrain University, Baghdad, Iraq between October 2016_ January2017 and control group comprised of 34 fertile women with at least 2 live births.the age of patients and control was (20-35) years.

Samples collection

Five milliliters of blood samples were collected from both ICSI patients at the time of embryo transfer and healthy control, 3 ml of blood were put into serum separating tube (SST) and centrifuged at (3000 rpm), separated sera were kept at −20°C for measurement of serum VEGF and about 2 ml of the blood samples were put into EDTA tube and kept at −20°C for DNA extraction and subsequent analysis of polymorphism using PCR-sequences.

Serum VEGF measurement

Samples were tested for VEGF protien using human VEGF sandwich enzyme –linked immunosorbent assay (ELISA, GenAsia, Chain) by manufacturer instruction. This assay has a detection limit range(0.02-6)mg/ml and the sensitivity of the assay was (0.104mg /ml).

Genotyping method

Genomic DNA was isolated from samples of whole blood using DNA isolation kit (*Genaid, Taiwan).* Genotyping was determined using polymerase chain reaction -sequence(PCR-sequence) method and the primers' sequences for the detection of VEGFA/rs 3025039 gene polymorphism are (F5'-AAGGAAGAGGAGACTCTGCGC-3) (R5'-TATGTGGGTGGGTGTGTCTACAG-3)[11] and OXTR/ rs53576 gene) polymorphism 23,24 are shown in table The PCR conditions were as follows: 5 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30sec, extension at 72ºC for 30sec, and a final extension at 72ºC for 10 min. the size of PCR products were 198 bp which detected on a 2% agarose gel. subsequently the PCR products for 3' untranslated region of VEGF-A gene were sent for sequencing to Macrogen Company (USA). Then, the sequencing for these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard (VEGF-A/rs 3025039) gene, using (Bioedit) software.

Statistical analysis

Statistical analysis Statistical package for the social sciences (SPSS), version 17.1 for windows software (SPSS inc., Chicago, USA) was used for statistical analysis. The data normally distributed and were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) hoc test was used to compare the parameters among groups.

RESULTS

VEGF serum levels

After follow up, patients divided in to three groups (pregnant, non-pregnant, miscarriage). In table (1) results showed that Women who underwent ICSI had significant elevated serum VEGF levels (P=0.0005) compared with control. Patients who received embryos and became pregnant had significant elevated serum VEGF levels (P=0.0004) when compared with control. VEGF level showed a non-significant (p= 0.3842) increase in patient who had implantation failure (non-pregnant) compared with control. Also there is no significant increase in miscarriage patients (p=0.1533) when compared with control. On the other hand pregnant group had significant elevated serum VEGF levels (P=0.0081) compared with those who did not become pregnant, while there is no significant difference between pregnant and miscarriage groups (p=0.8761) and also there is no significant difference between non pregnant and miscarriage groups (p=0.5972).

Table (1): VEGF level in control, ICSI patients,pregnant, non-pregnant, miscarriage.

	Group	mean±SD.	Pa	Pb	Pc	Pd	Pe	Pf	Pg
VEGF Mg/ml	Control N=34	1.99±0.54	0.0005	0.0004	0.3842	0.1533	0.0081	0.8761	0.5972
	ICSI Patient N=51	2.46±0.64							
	Pregnant N=19	2.80±0.61							
	Non pregnant N=27	2.21±0.60							
	Miscarriage N=5	2.56±0.30							

P^a value between control and ICSI patient

P^b value between control and pregnant

P^c value Between control and non-pregnant

P^d value between control and miscarriage

P^e value between pregnant and non-pregnant

P^f value between pregnant and miscarriage

P^g value between non-pregnant and miscarriage

n - sample size, SD - standard deviation

Genotypes frequencies for VEFGA/rs 3025039

The data of sequences indicated that the frequency of VEGFA/rs3025039 in the control group was C/C (85.29%) genotype followed by C/T genotype (11.76%) and (2.94 %) for T/T genotype. In whole ICSI patients, the highest genotype was the C/C (72.54%) followed by C/T (23.52%) and T/T genotypes (3.92 %). Patients divided in to 3 groups (pregnant, non-pregnant, miscarriage). In pregnant group, it was demonstrated that the highest genotype was C/C (62.5%) followed by C/T genotype (16.66%) and (0%) for T/T genotype. In non-pregnant group, the highest genotype was the C/C (70%) followed by C/T (25.92%) and T/T genotypes (3.7%). In miscarriage group, it was demonstrated that the highest genotype was C/C (60%) followed by C/T genotype (20%) and (20 %) for T/T genotype as showed in table.2.

By using Chi square test, it was also demonstrated that there were non-significant differences in VEGFA gene C, T polymorphism between ICSI patients groups and control group.

Table (2): The distribution of rs 3025039 genotype in the control and ICSI patients groups.

	Genotype frequency			Allele frequency		Pearson chi-square
	C/C	C/T	T/T	C	T	
Control (n=34)	29 (85.29)	4 (11.76)	1 (2.94)	0.9118	0.0882	2.4569
IVF patient (n=51)	37 (72.54)	12 (23.52)	2 (3.92)	0.8431	0.1569	0.6223
Pregnant (n=24)	15 (62.5)	4 (16.66)	0 (0)	0.8947	0.1053	0.263
Non-pregnant (n=27)	19 (70)	7 (25.92)	1 (3.70)	0.8333	0.1667	0.12
Miscarriage (n=5)	3 (60)	1 (20)	1 (20)	0.7	0.3	1.3719

As shown in table (3), the odds ratios of 936 C/T were calculated by comparison of control individuals and ICSI patients groups and the following results were obtained. The odds ratio for the 936 C/T was 2.35 and CI (0.68-8.0) with P value (P=0.17) the difference from the control was statistically non-significant, p>0.05. As well as, the 936 TT revealed a statistically non-significant difference from the control with an odds ratio was 1.56 and CI (0.13-18.1) with P value (P=0.71). In comparison between pregnant and control group, odd ratio for the TT genotype was 0.63 and CI (0.02-16.51) with non-significant (P=0.78) and the odd ratio for the CT genotype was 1.93 and CI (0.42-8.83) with non-significant (P=0.39). In

non-pregnant group, the odds ratio for the 936 CT was 2.6 and CI (0.68-10.38) The difference from the control was statistically non-significant, p>0.05 (p=0.15). As well, the 936 TT revealed a statistically non-significant difference from the control with an odds ratio was 1.52 and CI (0.08-25.90); p=0.76. On the other hand, the O.R was 9.66 and CI (0.47-197.2) when comparing the 936 TT with the CC of both miscarriages group and control group. There is no significant P>0.05 (P=0.14). The O.R was 2.41and CI was (0.19-29.23) when comparing CT with CC and P value was P=2.41 and also there is no significant difference.

Table (3): Comparison between genotypes.

936 C/T Polymorphism	Frequencies %		P value	OD & (95% CI)
	Control (n=34)	ICSI patients (n=51)		
CC	85.29(n=29)	72.54(n=37)	___	1.00(reference)
CT	11.76(n=4)	23.52(n=12)	0.17	2.35(0.68-8.0)
TT	2.94(n=1)	3.92(n=2)	0.71	1.56(0.13-18.1)
	Control(n=34)	**Pregnant(n=19)**	**P value**	**OD& (95% CI)**
CC	85.29(n=29)	62.5(n=15)	____	1.00(references)
CT	11.76(n=4)	16.66(n=4)	0.39	1.93(0.42-8.83)
TT	2.94(n=1)	0 (n=0)	0.78	0.63(0.02-16.51)
	Control(n=34)	**Non pregnant(n=27)**	**P value**	**OD & (95% CI)**
CC	85.29(n=29)	70 (n=19)	___	1.00(reference)
CT	11.76(n=4)	25.92 (n=7)	0.15	2.6(0.68-10.38)
TT	2.94(n=1)	3.7(n=1)	0.76	1.52(0.08-25.90)
	control	**Miscarriage (n=5)**	**P value**	**OD & (95% CI)**
CC	85.29(n=29)	60 (n=3)	____	1.00(reference)
CT	11.76(n=4)	20 (n=1)	0.48	2.41(0.19-29.23)
TT	2.94(n=1)	20 (n=1)	0.14	9.66(0.47-197.29)

OR: Odd Ratio, CI: confidence interval.

DISCUSSION

In this study the results showed that pregnant women have significant higher serum level (P>0.05) when compared with control. This result agree with Pang et al., 2013[12] but his samples were taken with women does not undergoing ICSI,Also the increase in pregnant group associated with the rate of good quality embryo as mentioned by Gao etal.,2012.[13]

lee et al.(1997)[14] said that at 11 to 14 days after embryo transfer in women undergoing ICSI, pregnant recipients of fresh embryos had higher serum VEGF levels than non-pregnant

recipients of fresh embryo. And this is agree with our result when compare between pregnant and non-pregnant groups (p>0.05).

In miscarriage group, there is no significant difference when compared with control or with pregnant group (P <0.05),this result disagreed with Almawi et al(2013)[15] who proved that the level of VEGF serum was significantly less in miscarriage cases than in control women. Recurrent implantation failure (RIF) can be defined as a clinical phenomenon that refers to a situation when, after the transfer of embryos, the implantation has repeatedly failed to reach a stage recognizable by ultrasonographic evidence of an intrauterine gestational sac.[16] In 'in vitro fertilization' (IVF) protocols, the implantation rate is approximately 25–40% with unsuccessful cases commonly being associated with RIF.[16]

VEGF is the best-characterized regulator of angiogenesis, an essential process for successful embryo implantation, since inadequate angiogenesis is related to implantation failure.[17] Overexpression or under expression of VEGF that encodes the proteins necessary for successful implantation may be a reason for decreased endometrial receptivity.[18] We have investigated whether the association of VEGFA variant 936C/T of the maternal genome with implantation failure. Results obtained demonstrated that the 936 C/T variant does not associate with implantation failure, This was the first study linking the 936C/T genetic variant with implantation failure.

Miscarriage has been associated with multiple causes, but in almost half of the cases, etiologies remain unknown. It is defined as the loss of pregnancies prior to the 20th week of gestation and affects 5% of couples[19, 20] human studies have proved a central role of VEGF in the maintenance of fetal and placental vasculature.[5,21,22] Stimulation of angiogenesis resulting from increased placental VEGFA gene expression is protective of the growing fetus,[23] and reduced VEGF expression was linked with defective embryogenesis and fetal loss[5,24] and first trimester trophoblast VEGF expression was weaker in placental samples from miscarriage cases than in control placenta.[21,25] It has been documented in several studies that rs 3025039, a single nucleotide polymorphism (SNP) in the 3' un translated region of VEGF gene, affect the expression of the gene, It has been shown that the 936 C/T polymorphism is correlated with a reduction in gene expression and that it drastically decreases plasma VEGF levels in VEGF 936 T allele carriers.

This effect was thought to be due to the loss of a specific region of interaction between VEGF and angiopoetin 4 or due to post-transcriptional regulation, leading to a transformation of the 3'-UTR, which may prolong the half-life of the mRNA.[26, 27] In our study, the genotype frequency do not significantly different between control and miscarriage groups.

Greek (Papazoglou et al. (2005),[28] Turkey (Şamlı etal.2012)[11] and Korean (Lee et al., 2010)[29] have reported similar results for this polymorphism, but in disagreement with the studies on north Indians (Aggarwal et al., 2011)[30] where an association between Miscarriage and 936C/T was reported. this discrepancies may be due to differences in population ethnic background and low numbers of study subjects.

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